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Studies on Age-related Diseases in Cultured Skin Fibroblasts

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In order to trace the origins of age-dependent diseases to the cellular level, we studied cultured human fibroblasts from subjects with 3 discrete inherited disorders and normal controls of various ages. Skin fibroblasts from subjects with progeria and Werner syndrome had a moderate to severe reduction in growth capacity, whereas cells from subjects with diabetes mellitus had a more subtle growth impairment. There was a decreased response of progeric fibroblasts to insulin-like hormones, and in normal cells the response decreased as a function of the passage level and donor age. Tissue factor, a procoagulant, was more abundant in progeric and Werner fibroblasts. An understanding of fibroblast aging *in vitro* may help us explain various concomitant phenomena of organismic aging such as diabetes mellitus, cell dropout, impaired hormone responsiveness, and increased atherothrombosis.

Numerous pathologic conditions accompany biological aging, and these include diabetes mellitus, atherosclerosis, and cancer [1]. The studies performed in our laboratory have primarily involved an *in vitro* approach to inherited human disorders of premature aging that feature early onset and accelerated progression of these age-associated conditions [2,3].

In this report I progress along a hierarchy from specific clinical entities to biochemical lesions in cells. I describe the growth of cultured fibroblasts, the response of fibroblasts to hormones with insulin-like activity (ILA), and finally some characteristics of intracellular enzymes and clotting factors. Alterations occur in all of these cellular factors as a function of donor age, whether in the usual sense of chronological age or in the sense of the advanced physiological aging of specific inherited disorders. Alterations also occur as a function of culture passage level, i.e., aging *in vitro* [4].

HUTCHINSON-GILFORD AND WERNER SYNDROMES

We have sought to exploit these 2 disorders in the study of biological aging for one major reason. Because each appears to involve a single gene (perhaps autosomal recessive in the Hutchinson-Gilford [progeria] syndrome and very likely autosomal recessive in the Werner syndrome), all clinical manifestations of premature aging should ultimately be traced to a single faulty gene product, i.e., a protein, and we should be able to distinguish between the primary disordered gene and the ensuing secondary phenomena. These disorders have been described in detail, and contrasts have been made to normal biological aging [2,5,6]. In brief, progeria is manifested during infancy as severely stunted growth, premature aged appearance, early onset of mesenchymal tissue degeneration, greying and loss of hair, and severe resistance to the hypoglycemic effect of exogenous insulin. Werner syndrome features similar problems although the onset age is characteristically beyond puberty. Additionally, overt diabetes of an insulin-resistant form is common in Werner syndrome, as are lenticular cataracts, skin ulcers, and severe osteoporosis. Despite these clear-cut differences, each disorder usually terminates with widespread atherosclerosis that ultimately leads to premature death through coronary or cerebrovascular occlusion. With Werner syndrome there is also a peculiarly high incidence of sarcomatous tumors.

DIABETES MELLITUS

In the more common forms of diabetes mellitus not associated with specific genetic syndromes, there is a decreased life expectancy even with insulin therapy [2,3,7]. The genetics of diabetes remains controversial, but it is becoming evident that this is a heterogeneous disease complex that involves many genetic and environmental variables, that is, multifactorial inheritance.

CURTAILED REPLICATIVE LIFE SPAN OF FIBROBLASTS

Cultured skin fibroblasts from both progeria and Werner syndrome patients have shown reduced replicative life spans (Table I), although more heterogeneous growth performances have been observed in fibroblasts from progeria patients. The normal mean of 50.3 mean population doublings (MPD) represents a large range in growth capacities because of the inverse correlation that exists between donor age and culture life span [8–12]. Progeria occurs in younger individuals, and their fibroblasts should in fairness be compared to age-matched control fibroblasts that perform somewhat better than the normal mean. Conversely, the more uniform growth depression apparent in Werner fibroblasts is due to their more advanced chronological and physiological age but may also be related to the fact that biopsy specimens have been taken from these individuals in later disease stages.

In the common forms of diabetes mellitus there is also reduced growth of cultured cells, but this phenomenon is more subtle. In coded studies on cultures derived from juvenile-onset diabetics, maturity-onset diabetics, and people genetically predisposed to the disease (both parents overtly diabetic ["prediabetic"]), there was an overall impairment of growth that began at the stage of primary cultures (Table II). In these same 3 groups bearing the diabetic genotype, growth was also impaired in secondary cultures (Fig 1). The growth advantage of normal cultures was relatively small and only reached statistical significance in some cases. However, the trends were reproducible over the first 7 subcultures, an indication that the diabetic growth deficit is a persistent heritable characteristic. The effect of advancing culture age was seen in fibroblasts from these 3 groups and in those from controls as the lengthening interval required to attain confluence.

The onset of senescence also occurred earlier in cultures emanating from prediabetic and diabetic donors (Table III). The replicative life span (Table III) was marginally longer in normal cells, and there was an apparent downward progression through prediabetic and diabetic cultures. These differences, however, did not reach statistical significance. I must emphasize that control cultures were derived from normal individuals who were stringently selected on the basis of negative family histories for diabetes and on the basis of repeatedly normal results of glucose tolerance tests. Two further points are worth empha-

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Abbreviations:
ILA—insulin-like activity
MPD—mean population doublings

TABLE I. *Replicative life span of cultured fibroblasts from skin of subjects with progeria, Werner syndrome, and controls*[a]

Donor group	Donor age (yr)	Replicative life span (mean population doublings)
Controls		
85 subjects	newborn—75 years	50.3 ± 2.1[c]
Progeria[b]		
Case 1	2.5	36
Case 2	6	52
Case 3	9	6
Case 4	9	37
Werner syndrome[b]		
Case 1	37	12
Case 2	43	5
Case 3	46	12
Case 4	48	8
Case 5	49	6

[a] Data from reference 2.
[b] There are several reports not included here of primary growth failure in progeria and Werner cultures.
[c] Mean ± SD.

TABLE II. *Intensity of outgrowth at late stages of primary culture*[a]

Donor group	First plateau[b] (days)
Normal	13.24 ± 0.63
Prediabetic	15.45 ± 0.69
Juvenile-onset diabetics	17.41 ± 0.84
Maturity-onset diabetics	18.21 ± 1.14

[a] Mean ± SE. Normal versus prediabetics, $p < .05$; normal versus juvenile-onset diabetics, $p < .01$; normal versus maturity-onset diabetics, $p < .01$. From reference 13.
[b] Earliest time in days at which the maximum percentage of explanted skin fragments showed growth.

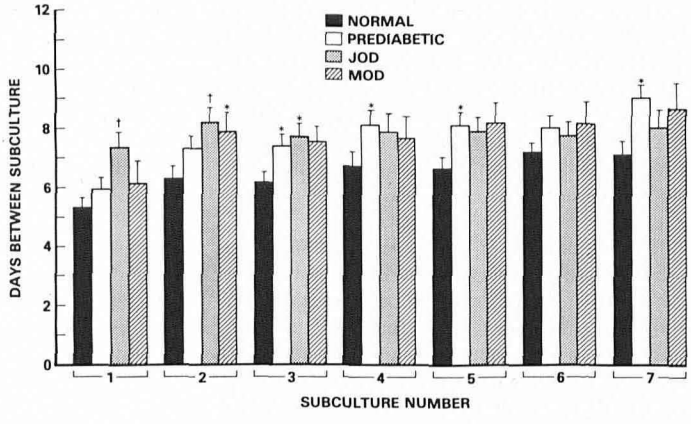

FIG 1. Growth vigor of secondary fibroblast cultures measured as the time required to attain confluence after subculture. A shorter interval indicates greater vigor. Data are the means ± SEM. Statistically significant differences versus normal are * $p < .05$ and † $p < .01$. *JOD* = juvenile-onset diabetes; *MDD* = maturity-onset diabetes. From reference 13, reproduced with permission of The Rockefeller University Press.

sizing. First, the overall growth of a culture is sustained by the most vigorous subpopulation of cells. Thus, these diabetic cultures may already have had a greater fraction of senescent cells that were unresponsive to growth factors in the culture medium even though overall capacity to proliferate appeared only marginally impaired. In fact, there is direct evidence for such heterogeneity in all 3 diabetic groups discussed here [13] and in normal individuals [14]. Second, during establishment of cultures, only the most vigorous cells grow out of primary skin explants, and we chose to propagate secondary cultures using only the best outgrowths from several replicate dishes. Hence, it is likely that we introduced some bias against the demonstration of impaired fibroblast growth *in vitro*. It may be that a greater proportion of diabetic and prediabetic cells are senescent *in vivo*.

DECREASED RESPONSE OF CELLS TO INSULIN-LIKE ACTIVITY DURING AGING *IN VIVO* AND *IN VITRO*

Many attempts to study the effects of insulin in cultured human fibroblasts have been hampered by both sluggish responses and the need to carry out tedious manipulations, e.g., serum starvation for 24 hr. These problems are probably related to the fact that insulin, in contrast to the many insulin-like polypeptides recently elucidated, is not the prime stimulating hormone for fibroblasts [15]. A number of these growth factors share sequence hemology with insulin and have overlapping receptor reactivity [16]. Recent work in our laboratory [17,18] has shown that fibroblasts derived from young normal donors need relatively small amounts of ILA for the stimulation of DNA synthesis (Fig 2). The concentration of ILA required to

TABLE III. *Onset of senescence and replicative life span of cultured skin fibroblasts from normal, prediabetic, juvenile-onset, and maturity-onset diabetic donors*[a]

Donor group	Onset of senescence (MPD to first refeeding)	Replicative life span (MPD to termination)
Normal	23.9 ± 2.9[b]	52.5 ± 2.2[c]
Prediabetic	12.6 ± 3.1	47.8 ± 2.4
Juvenile-onset diabetics	12.4 ± 3.8	47.1 ± 3.0
Maturity-onset diabetics	13.5 ± 5.2	46.4 ± 4.0

[a] Mean ± SE mean population doublings (MPD). Data are from reference 13.
[b] Normal versus prediabetics, $p < .05$; normal versus juvenile-onset diabetics, $p < .05$.
[c] Differences between normal and other 3 groups: not significant.

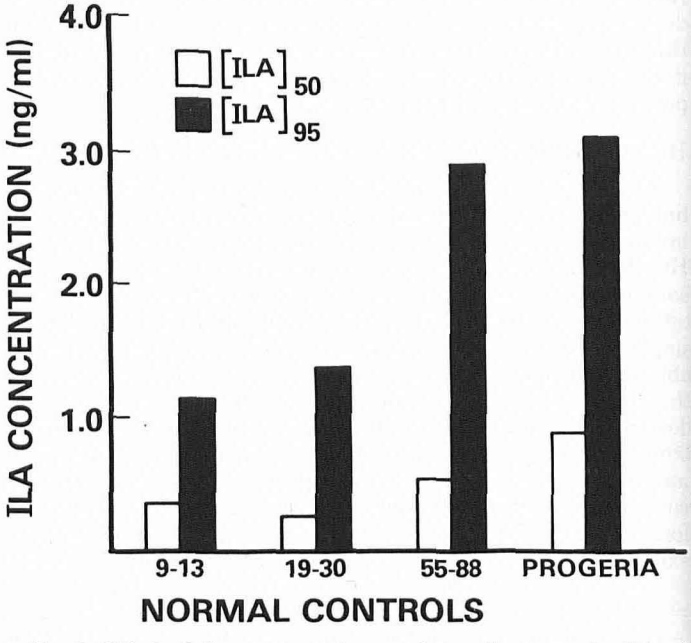

FIG 2. Effect of donor age and progeria on the concentration of insulin-like activity (*ILA*) required to stimulate DNA synthesis to 50% and 95% of the maximum response. DNA synthesis was measured by ^{3}H-thymidine incorporation into trichloracetic-acid-precipitable material. The age range of controls is given below the *bars*. The progeria subject was 9 yr old. From reference 18 and C. B. Harley et al, in preparation.

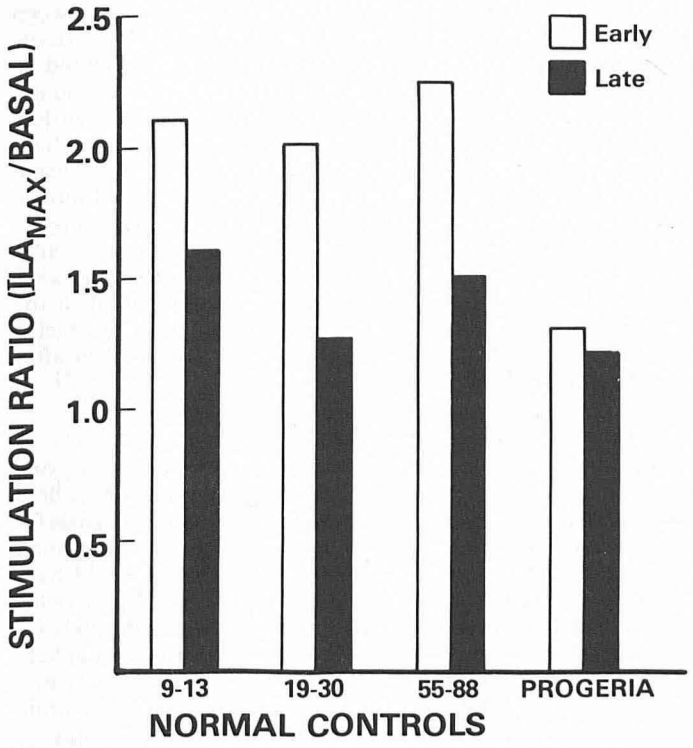

FIG 3. Effect of aging *in vitro* on maximum insulin-like activity (*ILA*) stimulation of 2-deoxyglucose uptake. Fibroblasts were allowed to grow to confluence. They were then rinsed and incubated for 3 hr with a maximal stimulating concentration of ILA (4 ng equivalents/ml with respect to a radioreceptor assay for insulin [ref 48]) or normal saline (basal) and then incubated in medium without glucose containing ^{3}H-2-deoxyglucose. After a series of times up to 10 min, the rates of radioactivity accumulation in acid-insoluble material were determined, and the ratio of ILA-stimulated uptake to basal uptake was calculated. From reference 18 and C. B. Harley et al, in preparation.

produce 50% of the maximum stimulation was only slightly greater in the oldest normal donors. On the other hand, the ILA concentration required to produce 95% of the maximum stimulation was substantially greater in this older group. Progeria cells are clearly unique since they require even greater amounts of ILA for stimulation at both the 50% and 95% levels than do cells of the oldest group of controls. A similar increase in ILA_{50} and ILA_{95} also occurs as a function of the passage level [17,18].

Using 2-deoxyglucose, an analogue of glucose (the most commonly measured parameter in diabetes), we also found that the ratio of maximum hormone-stimulated uptake to basal uptake was consistently decreased in all age groups as cultures traversed the *in vitro* life span (Fig 3). Progeria cultures showed an even greater reduction in the stimulation ratio, but the difference in these cells between early and late passage was small probably as a result of the restricted replicative life span. This lower stimulation ratio in progeria is in part attributable to a higher basal level of uptake [17,18]. The same effect has been observed in late-passage normal fibroblasts (Table IV), and it appears that aged cells are relatively unable to "down-regulate" their carbohydrate metabolism [18] and protein metabolism (C. B. Harley et al, in preparation). Thus, the basal uptake of glucose is higher, the maximum stimulation by ILA is lower, and a narrow excursion results.

The reasons for this impaired responsiveness to ILA may reside anywhere in the sequence from the hormone receptor on the cell surface, to the purine cyclase systems in the plasma membrane, to the intracellular enzymatic machinery. Although we have no data on ILA receptors in cultured fibroblasts, our studies have shown that the insulin receptor is altered as a function of age [20,21]. With the many similarities between insulin and ILA polypeptides, we speculate that altered ILA receptors may be a prominent factor in the blunted response of aged cells to ILA. Indeed, a recent report by Villee and Powers [22] has confirmed that skin fibroblasts from the same progeric patient we studied were resistant to insulin stimulation of amino acid and glucose incorporation into proteins and glycogen, respectively.

AGE-DEPENDENT CHANGES IN INTRACELLULAR ENZYMES

Impaired hormone responsiveness of cells may also be related to altered enzymatic machinery distal to the membrane receptor. Although the findings for normal cultures aging in vitro have been controversial [23–26], the results in progeria and Werner syndrome have been more clear-cut; rather consistent and relatively large heat-labile subfractions have been found in cultured fibroblasts of both progeria and Werner syndrome patients [27–30] and in circulating erythrocytes [31,32]. Observation of erythrocytes (Table V) freshly removed from the circulation has substantiated the idea that heat-labile enzymes *in vitro* reflect a valid biological phenomenon. The exact structural basis for these alterations remains unknown, but we are exploring various possibilities [33,34]. These include primary changes in the amino acid sequence of enzymes introduced at the level of messenger RNA translation, posttranslational alterations, and problems at the level of DNA or RNA metabo-

TABLE IV. *Glucose consumed during 2-day intervals of growth to confluence in early- and late-passage cultures (nanograms per cell)*[a]

Days	Passage level		Ratio of L/E
	Early	Late	
0–2	6.1	16.0	2.6
2–4	2.3	6.0	2.6
4–6	1.5	3.6	2.4
6–8	1.3	2.6	2.0
8–10	0.9	2.3	2.6
10–12	0.6	2.0	3.3

[a] Early- and late-passage cultures became confluent on days 6 and 10, respectively. From reference 19.
Abbreviation: L/E = late/early.

TABLE V. *Fraction of heat-labile enzymes in circulating erythrocytes*[a]

Enzyme	Heat-labile fraction[b]	p value[c]
Progeria family		
Proband		
G6PD	22.26 ± 4.33 (5)	.001
6PGD	22.31 ± 8.09 (3)	.005
HGPRT	21.20 ± 1.84 (2)	.05
Mother		
G6PD	13.83 ± 3.79 (5)	.001
6PGD	9.13 ± 2.58 (2)	.1
HGPRT	19.69 ± 4.04 (3)	.1
Father		
G6PD	11.57 ± 1.36 (4)	.001
6PGD	9.69 ± 1.84 (2)	.05
HGPRT	28.05 ± 4.76 (3)	.02
Controls		
G6PD	3.43 ± 0.75 (8)	
6PGD	5.05 ± 1.40 (6)	
HGPRT	11.68 ± 4.41 (5)	

[a] Data are mean percent ± SEM. Data are from reference 32.
[b] Numbers of experiments on separate blood samples are indicated in parentheses.
[c] Significance of difference: family member versus controls; $p <$ values are shown.
Abbreviations: G6PD = glucose-6-phosphate dehydrogenase; 6PGD = 6-phosphogluconate dehydrogenase; HGPRT = hypoxanthine-guanine phosphoribosyl transferase.

lism. Whatever the basis may be, enzymes affected in this way probably are less efficient catalytically in response to substrates, cofactors, and other small molecules concerned with regulation. These facts, combined with the results of earlier studies on cyclic AMP [35–37], indicate that alterations may occur at each step in the sequence from hormone receptor through the cyclic nucleotide system and intracellular enzymes. In concert, all of these steps may be responsible for the blunted response of aging cells to hormones like insulin. An exciting recent report by Kobayashi et al [38] indicates that in Donohue syndrome (i.e., leprechaunism, which shares many features, such as severe growth stunting and insulin resistance, with progeria) insulin binding is normal but the impaired responsiveness of cultured cells with respect to glucose uptake resides in a postreceptor defect, perhaps in the cell membrane or the distal enzymatic machinery.

INCREASED TISSUE FACTOR CONTENT IN PROGERIA AND WERNER FIBROBLASTS

The complex scheme of blood coagulation can be reduced to 2 major pathways, the intrinsic and extrinsic [39]. Both can be triggered by initial interaction with components of the cell wall *in vivo*, but each can be analyzed individually in test tubes. In brief, the intrinsic system involves circulating protein factors, primarily factor XII (Hageman factor), and factor VIII (antihemophilic factor). The extrinsic system involves tissue factor and factor VII, which "short-circuits" many of the reactions in the intrinsic system that result in thrombin production. This reaction in turn commences the polymerization of fibrinogen to the mature fibrin clot.

We observed that tissue factor activity was consistently higher in cultured fibroblasts from progeria and Werner subjects (Table VI) and ranged from 23 units to 137 units. The site of the skin biopsy did not seem to influence this activity. Normal control cells showed no effect of chronological age over the span of 5 to 75 years, and the effect of in vitro passage appeared to increase the mean tissue factor level (Table VI). This increase was not statistically significant.

Fibroblast tissue factor was specific, that is, through factor VII, for the extrinsic clotting mechanism. Thus, when cells were incubated prior to assay with specific immunoglobulins directed against purified placental tissue factor, both normal and progeria cells demonstrated progressive prolongation of the clotting time; prolongation did not occur after treatment with preimmune globulins or normal saline. Cells disrupted by sonication showed an approximately 5-fold increase in tissue factor activity in all normal and abnormal strains, but the differences between these cells remained the same. Thus, higher levels of tissue factor in aging syndrome fibroblasts appear to be related to increased content rather than increased availability. Although we are unsure why cells from 2 genetically different disorders show similar elevations in tissue factor activity, the long-held concept of Rokitansky [41], who proposed that coagulation mechanisms acting inwardly upon arterial walls contribute to atherosclerosis, is noteworthy. Plasma membranes of cells within the arterial intima and media layers appear to be rich in tissue factor antigen in both the large blood vessels and vasa vasorum [42]. Moreover, tissue factor is also abundant in the vicinity of atheromatous plaques. It seems clear that this factor can initiate coagulation after plaque disruption or even after minimum cell damage.

TABLE VI. *Tissue factor activity of cultured skin fibroblasts*[a]

Donor group	Donor age (yr)	Tissue factor (units)
Control		
Early passage	range 5–75	5.06 ± 3.84[b]
Late passage		7.96 ± 6.84
Progeria		
Case 1	2.5	42
Case 2	9	126
Case 3	9	
Anterior forearm		23
Anterior thigh		47
Left calf		137
Right calf		109
Werner syndrome	56	46

[a] All cultures were derived from the skin of the anterior forearms of 10 normal donors and of persons with progeria and Werner syndrome, except where otherwise indicated. From reference 40. Early-passage normal cells were assayed in the first half of their replicative life span, which ranged from 48 to 75 mean population doublings (MPD) with late-passage cells at 3 to 9 MPD before termination. Progeria cells (replicative life spans, range 21 to 40 MPD) were assayed with 9 to 30 MPD remaining and Werner cells (life span 15 MPD) within 3 to 6 MPD of termination.

[b] Mean ± SD.

CONCLUSIONS

Studies on cultured fibroblasts undergoing aging *in vivo* and *in vitro* give insight into several mechanisms of age-dependent disease. Senescent cell dropout may be the underlying basis for the observed depletion of stem cell compartments in poorly renewable systems *in vivo*. A contracted cell mass would have a reduced functional capacity, and we speculate that pancreatic β cell dropout frequently occurs in insulin-dependent diabetes whether the inciting agent is a virus, a toxin, or even a genetically determined limitation of the regenerative response. Similarly, premature cellular dropout in specific regions of the brain in both senile dementia and Huntington disease [43] may be related to neuronspecific defects in gene expression, susceptibility to exogenous agents, or both.

A progressively diminished responsiveness to insulin and related hormones occurs during aging *in vitro*, and this phenomenon appears to be correlated with the rising incidence of diabetes mellitus in aging populations [44]. Most people with maturity-onset diabetes do not lack insulin; indeed, their circulating levels of this hormone are often higher than normal. The problem, certainly in the insulin resistance seen in progeria and Werner syndrome, appears to be a reduced responsiveness [45]. It is not improbable that the growth defects in the fibroblasts of progeria, Werner syndrome, diabetic, and prediabetic individuals are in great part due to the poor response of these fibroblasts to insulin and insulin-like serum factors in the growth medium.

However, age-dependent changes are not always decremental since we and others have shown that epinephrine-induced rises in cyclic AMP concentration occur in aged fibroblasts at the same time that prostaglandin E_1 stimulation falls [35–37]. The divergent responses to these 2 hormones are not understood, but they are probably mediated through mechanisms different from those of polypeptide hormones. Further studies should help illuminate the basis of aberrant responses to adrenergic hormones in the elderly as well as the idiosyncratic drug reactions frequently observed.

Finally, although tissue factor does not increase significantly in aged normal fibroblasts as it does in Werner and progeria fibroblasts, clonal heterogeneity must be borne in mind. One need only postulate a small patch of aged vascular cells in the arterial or venous tree capable of leading to an atherothrombotic event. Moreover, if acting in concert with the impaired clot retraction previously described [46,47], this important group of vascular degenerative diseases could emanate from the cellular fixed tissue compartment and would not have to arise solely as a consequence of abnormal circulating clotting factors. This system of cultured skin fibroblasts should play a central role in helping us unravel the mysteries of age-dependent diseases and should lead to rational plans for prevention and therapy.

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